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CARBON DIOXIDE FIXATION AND GLYCOGENESIS IN THE FLATWORM
HYMENOLEPIS DIMINUTA

by

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Abstract

The cyclophyllidean cestode *Hymenolepis diminuta* has been found to incorporate $^{14}\text{C}_2\text{O}_2$ into organic acids, amino acids, and glycogen upon *in vivo* incubation. This incorporation was sensitive to incubation conditions, more $^{14}\text{C}_2\text{O}_2$ being incorporated aerobically than anaerobically, and was also stimulated by starvation of the worms. The fact that no $\alpha$-ketoglutarate could be detected and that no $^{14}\text{C}$ could be observed in glutamic acid upon incubation with $^{14}\text{C}_2\text{O}_2$ indicated the absence of a complete citric acid cycle in this worm. There did appear to be an energy dependent connecting link between the glycolytic sequence and the dicarboxylic acid shuttle.

An active phosphoenolpyruvate carboxylase, similar to the one present in avian liver, was found to be present in *H. diminuta*. This activity was stimulated by starvation and was abundant enough to account for the *in vivo* incubation results.

A low level of malic enzyme activity was also found in acetone powder extracts of the worm. It was too low in activity to be detected in spectrophotometric assays, but was studied through the measurement of $^{14}\text{C}_2\text{O}_2$ incorporation into L-malate. From these preliminary studies the enzyme did not appear abundant enough to be physiologically significant.
A third CO$_2$ fixation activity was found in the acetone powder extracts. It appeared to be different from any enzyme yet found and required magnesium ion and coenzyme A for activity. No nucleotide requirement could be demonstrated. Fixation was found to result in malate. The enzyme has been tentatively named the "malate synthesizing enzyme."

From this study it would seem very likely that carbon dioxide fixation may play a central role in glycogenesis and carbohydrate metabolism in *Hymenolepis diminuta*. 
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CARBON DIOXIDE FIXATION AND GLYCOGENESIS IN THE FLATWORM
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Introduction

Carbon dioxide fixation by heterotrophic organisms was discovered by Wood and Werkman (1936) while studying the fermentation of glycerol by propionic acid bacteria. The amounts of succinate formed and carbon dioxide used were found to be approximately equimolar (Wood and Werkman, 1938). Wood and Werkman (1940) proposed that pyruvate and CO$_2$ formed oxalacetate which was then reduced to succinate. The CO$_2$ fixation reaction leading from pyruvate to the C$_4$-dicarboxylic acids was subsequently referred to as the "Wood-Werkman reaction." This phenomenon has been found to occur in many animal tissues, microorganisms, and plants; and to be due to several fixation mechanisms.

Utter and Wood (1946) demonstrated an ATP requirement for the C$^{14}$O$_2$ incorporation into oxalacetate catalyzed by pigeon liver extracts and postulated the presence of a phosphorylated intermediate. Purification of the enzyme was finally achieved by Utter and Kurahashi (1954). The enzyme was found to be definitely separate from the malic enzyme and to catalyze reaction 1.

(1) phosphoenolpyruvate + CO$_2$ + IDP $\rightleftharpoons$ oxalacetate + ITP

ITP*, not ATP, was found to be effective in the reaction

*Abbreviations used in the text are the following: adenosine di- and triphosphate, ADP and ATP; p-chloromercuribenzoate, p-CMB; coenzyme A, CoA; diphosphopyridine nucleotide, DPN; ethylenediaminetetraacetic acid, EDTA; flavin-adenine dinucleotide, FAD; guanosine triphosphate, GTP; inosine triphosphate, ITP; phosphoenolpyruvate, PEP; reduced glutathione, GSH; triphosphopyridine nucleotide, TPN.
(Utter, Kurahashi, and Rose, 1954). However, GTP provided more stimulation at lower concentrations and therefore was thought to be the physiologically active nucleotide (Kurahashi et al., 1957). The yeast enzyme, unlike the pigeon liver enzyme, was found to use adenosine derivatives (Canna and Stoppani, 1959). The enzyme showed a sulfhydryl dependence for activity (Utter, Kurahashi, and Rose, 1954). It also required manganous or magnesium ions. Graves et al. (1956) demonstrated that the reaction does not result in the enol form of oxalacetate or a phosphate derivative. Utter, Kurahashi and Rose (1954) suggested that the mechanism may be the reverse of the mechanism proposed by Steinberger and Westheimer (1951) for the decarboxylation of the dimethyl derivative of oxalacetate (see Williams, 1953).

The malic enzyme was first discovered in pigeon liver by Ochoa et al. (1947, 1948). The enzyme required manganous ion for activity and was TPN-specific. It catalyzed the reversible oxidation and decarboxylation of L-malate to pyruvate and CO$_2$ according to reaction 2.

$$\text{L-malate} + \text{TPN} \rightleftharpoons \text{pyruvate} + \text{CO}_2 + \text{TPNH}$$

In the presence of manganous ion, the enzyme would also catalyze the decarboxylation of oxalacetate to pyruvate and CO$_2$. Magnesium ion could replace manganous ion in the reactions, but was less effective (Viega Salles and Ochoa, 1950). The pH optimum of the TPN-reduction activity was about 7.5; that of the oxalacetate decarboxylating activity was around 4.5. It was also demonstrated that malate
could readily be formed from pyruvate and CO₂ in the presence of a TPNH-generating system (Ochoa et al., 1950).

Flavin, Ochoa and coworkers (1957) in a series of studies found that rat liver and pig heart extracts contained an enzyme catalyzing the carboxylation of propionyl CoA to form methylmalonyl CoA. The carboxylation required magnesium ion and split orthophosphate from ATP. It was shown to contain biotin which was probably involved in the reaction (Kaziro et al., 1960; Kaziro and Ochoa, 1961). A methylmalonyl CoA isomerase converting methylmalonyl CoA to succinyl CoA was also found. Originally it did not appear to have any metal or cofactor requirements (Flavin and Ochoa, 1957; Beck et al., 1957; Beck and Ochoa, 1958). Recently, however, the enzyme has been shown to require a B₁₂ coenzyme (Gurnani, Mistry and Johnson, 1960; Lengyel, Mazumder and Ochoa, 1960) and to catalyze an intramolecular, rather than intermolecular, shift in its isomerization of methylmalonyl CoA to succinyl CoA (Phares, Long and Carson, 1962; Kellermeyer and Wood, 1962). Mazumder et al. (1961) discovered that the carboxylase produced only one enantiomorph, the one which the isomerase could not use. A methylmalonyl CoA racemase served to convert one enantiomorph to the other. The complete sequence (reaction 3) involved fixation into propionyl CoA to form methylmalonyl CoA. This was then racemized and the proper enantiomorph isomerized to succinyl CoA via an intramolecular shift.
(3) propionyl CoA + CO₂ + ATP ⇌ methylmalonyl CoA(a) 

\[ \text{succinyl CoA} \rightleftharpoons \text{methylmalonyl CoA(b)} \]

Recently Utter and Keech (1963) have isolated and partially purified an enzyme from chicken liver which catalyzes the formation of oxalacetate from pyruvate and CO₂ in the presence of ATP, magnesium ion, and acetyl CoA (reaction 4).

(4) pyruvate + CO₂ + ATP ⇌ oxalacetate + ADP + P_i 
The acetyl CoA serves only as a catalyst. Pyruvate carboxylase is a biotin enzyme and is inhibited by avidin and sulfhydryl inhibitors.

Bandurski and Greiner (1953) found an enzyme in acetone powders of spinach leaves which catalyzed the irreversible carboxylation of phosphoenolpyruvate to form oxalacetate and orthophosphate according to reaction 5.

(5) phosphoenolpyruvate + CO₂ ⇌ oxalacetate + P_i 
Magnesium ion and a reducing agent such as glutathione were required for the PEP carboxylase activity. No nucleotide requirement could be demonstrated. A similar activity was found in wheat germ (Tchen and Vennesland, 1955).

Siu et al. (1961), in a preliminary communication, reported the discovery of an enzyme from Propionibacterium shermanii which catalyzed reaction 6.

(6) phosphoenolpyruvate + CO₂ + P_i ⇌ oxalacetate + PPF_i 
The phosphoenolpyruvate carboxytransphorylase required either magnesium or manganous ions for activity.

Carbon dioxide fixation has long been thought to play a
part in glycogenesis via a reversal of glycolysis. Solomon et al. (1941) observed C\textsubscript{11} incorporation into liver glycogen following intraperitoneal injection of rats with NaHC\textsubscript{11}O\textsubscript{3}. They postulated that C\textsubscript{11}O\textsubscript{2} and pyruvate formed C\textsubscript{11}-labelled oxalacetate. The labelled carbon could be randomized in the dicarboxylic acid shuttle and then contribute to glycogenesis via phosphoenolpyruvate formation from oxalacetate. After the intraperitoneal injection of NaHC\textsubscript{13}O\textsubscript{3} into starved rats, the C\textsubscript{13} appeared in carbons 3 and 4 of the glucosyl unit of liver glycogen (Wood et al., 1945). This lent support to Solomon's hypothesis since carboxyl-C\textsubscript{13} labelled phosphoenolpyruvate would be expected to result in labelling of the carbons 3 and 4.

Previously pyruvic kinase had been thought to be irreversible. Lardy and Zeigler (1945) demonstrated the reversibility of this reaction by measurement of P\textsubscript{32} distribution and phosphoenolpyruvate formation. The reversal required potassium and magnesium. They proposed that glycogenesis from pyruvate or lactate could be accounted for by a simple reversal of the kinase reaction as well as fixation to form a C\textsubscript{4}-dicarboxylic acid.

The incubation of rabbit liver slices with pyruvate as a substrate and NaHC\textsubscript{14}O\textsubscript{3} resulted in the labelling of only carbons 3 and 4 of the glycosyl unit (Topper and Hastings, 1949). When pyruvate-2-C\textsubscript{14} was used, all carbons were labelled. The data indicated that the carboxylation of
pyruvate and subsequent equilibration in the dicarboxylic acid shuttle occurred about 4 times as fast as did the direct phosphorylation of pyruvate.

Utter and Kurahashi (1954) isolated phosphoenolpyruvate carboxykinase and obtained data indicating that the fixation route through the dicarboxylic acids to phosphoenolpyruvate was much more thermodynamically favorable than the reverse of the pyruvic kinase reaction. They suggested a pathway consisting of fixation into malate via the malic enzyme, randomization of the label in the dicarboxylic acid shuttle, and phosphoenolpyruvate formation from oxalacetate.

The feasibility of such a pathway was supported by the demonstration of phosphoenolpyruvate formation from citric acid cycle intermediates in pig liver mitochondria (Bandurski and Lipmann, 1956).

Hiatt et al. (1958) found C\textsuperscript{14} incorporation into rat liver and diaphragm glycogen upon incubation with either pyruvate-2-C\textsuperscript{14} or NaHCO\textsubscript{3}C\textsuperscript{14}O\textsubscript{3}. Pyruvate label was extensively randomized in the liver glucosyl units, but only present in carbons 4 and 5 of diaphragm glucosyl units. These results were interpreted as indicating pyruvate entrance into glycolysis via the dicarboxylic acid shuttle in the liver and through a reversal of the kinase reaction in the diaphragm muscle.

Almost all the phosphoenolpyruvate carboxykinase was found present in the kidney and liver of rats where most of
the glycogenesis from pyruvate and lactate occur (Utter, 1950). On the other hand, pyruvic kinase was about 40 times more active in the muscle than in the liver. The rat liver carboxykinase was also found to be sensitive to starvation and hormonal control (Shrago et al., 1963).

From these studies it would appear that carbon dioxide fixation into the dicarboxylic acids may well be extremely important in glycogenesis from pyruvate.

The enzymes involved in carbon dioxide fixation have been studied for the most part in vertebrates and bacteria. The study of the mechanism of fixation in invertebrates has been very limited. Hammen and Osborne (1959) found $^{14}O_2$ incorporation to occur in representatives of at least 12 marine phyla. Incorporation into the calcium carbonate shell matrix of the oyster *Crassostrea virginica* has also been observed (Hammen and Wilbur, 1959). *In vitro* incubations using mantle tissue and NaH$^{14}O_3$ resulted primarily in the incorporation of $^{14}C$ into succinate and aspartate. From these results they concluded that fixation resulted in succinate formation from propionate.

Awapara and Campbell (1964) investigated $^{14}O_2$ incorporation into the free amino acids of the oyster *Crassostrea virginica*, the land snail *Otala lactea*, and the brackish water clam *Rangia cuneata*. Incorporation into alanine, aspartate, and glutamate was observed in all three animals. The data indicated that fixation formed oxalacetate rather
than succinate as Hammen and Wilbur had suggested.

Carbon dioxide fixation has also been found in arthropods. Silkworms contain a TPN-requiring malic enzyme activity which shows no oxalacetic decarboxylase activity (Paulkner, 1956). Cheng and Waelsch (1962) found $\text{C}^{14}\text{O}_2$ incorporation into aspartate, glutamate, malate, and citrate by lobster nerve preparations. The major portion of the $\text{C}^{14}$ was found in aspartate.

The marine flatworms *Stylochus zebra* and *Bdelloura candida*, the planarian *Dugesia tigrina*, and the trematode *Entobdella bumpsi* were found to incorporate $\text{C}^{14}\text{O}_2$ into organic acids. (Hammen and Lum, 1962). The $\text{C}^{14}$ appeared originally in malate.

Only two studies have been made of CO$_2$ fixation in nematodes. Fairbairn (1954) found $\text{C}^{14}\text{O}_2$ incorporation into several compounds where *Heterakis gallinae* was incubated anaerobically with NaHC$_{14}$O$_3$. About two-thirds of the $\text{C}^{14}$ appeared in propionate and most of the remainder in an acid tentatively identified as succinate. The metabolism of this worm appeared to be quite similar to that of the bacterial genus *Propionibacterium*. Malic enzyme was discovered in *Ascaris lumbricoides* muscle by Saz and Hubbard (1957). It differed from the vertebrate enzyme in having a DPN preference and in having no oxalacetate decarboxylase activity. Succinate, the major fermentation product, was found to be formed by the carboxylation of pyruvate and subsequent reduction to succinate (Saz and Vidrine, 1959). The *Ascaris* muscle strips
were also capable of the reversible decarboxylation of succinate to propionate. However the carboxylation of propionate did not appear to be a major pathway. The electron transport system of *Ascaris* was also found to be different than that in vertebrates (Kmetic and Bueding, 1961). It consisted of several RAD flavoprotein carriers. Fumarate was observed to be rapidly reduced by a DPNH dehydrogenase coupled to succinic dehydrogenase. Since very little lactate is produced by intact *Ascaris*, the suggestion was made that the DPNH formed during glycolysis could be reoxidized by fumarate reduction to succinate and that anaerobic phosphorylation accompanied this process. *Ascaris* muscle sarcosomes were found to contain flavoprotein carriers, succinic and malic dehydrogenases, diaphorase, fumarase, and oxalacetate decarboxylase (Seidman and Entner, 1961). These enzymes were shown to be involved in a concerted anaerobic dismutation of malate and reduction of fumarate coupled to ATP formation (reaction 7).

\[
\begin{align*}
2 \text{malate} + \text{ADP} + \text{Pi} & \rightarrow \text{pyruvate} + \text{CO}_2 + \text{succinate} + \text{ATP} \\
\downarrow & \\
\text{fumarate} & 
\end{align*}
\]

This sequence would account for the observed succinate accumulation and the stimulation of DPNH oxidation by fumarate. As Fairbairn (1961) pointed out, a deprivation of CO\(_2\) would lead to a deficiency of fumarate (and malate) resulting in a retardation of DPNH oxidation and high-energy phosphate production. This would inhibit glycolysis due to the lack of DPN and glycogenesis due to the lack of high-energy phosphate.
Fairbairn suggested that a similar situation might exist with *Hymenolepis diminuta*.

Agosin and Repetto (1963) found $^{14}$O$_2$ incorporation into the tricarboxylic acid cycle intermediates and polysaccharides by intact scolices of the cestode, *Echinococcus granulosus*. The major portion of $^{14}$C appeared in succinate. Phosphoenolpyruvate carboxykinase and malic enzyme were found to be present. The main fixation route appeared to be via the carboxykinase reaction. The data also indicated that CO$_2$ fixation could effect glycogenesis through a reverse of glycolysis.

From the limited number of studies, it appears at the present time that carbon dioxide fixation is of fairly universal distribution among the invertebrates and probably of great metabolic importance.

The citric acid cycle, glycolysis, and glycogenesis have been thoroughly studied in the cyclophyllidean cestode, *Hymenolepis diminuta*. The worm was found to possess a cytochrome oxidase linked to succinic dehydrogenase (Read, 1952). Malic dehydrogenase and fumarase were also found (Read, 1953).

No evidence was obtained, however, for the presence of the enzymes catalyzing the oxidation of citrate and isocitrate (Read, 1953). Furthermore, only succinate and malate, of the citric acid cycle intermediates tested, either sustained or stimulated respiration in the worms (Read, 1956). These results indicated the absence of a complete citric
acid cycle in the worm.

The presence of the intermediates of glycolysis and several of the glycolytic enzymes provided strong evidence for the presence of a complete glycolytic pathway similar to that found in vertebrates (Read, 1951).

There is a great deal of evidence that cestodes require carbohydrate for normal growth and reproduction. Chandler (1943) observed a decrease in the number of *H. diminuta* becoming established in the rat host and also a decrease in the size of the resulting adults when the hosts were fed a carbohydrate deficient diet. More detailed studies demonstrated that both worm weight and egg production fell off when the hosts were either starved or fed carbohydrates such as sucrose and fructose (Read and Rothman, 1957b). A direct relation between the absolute quantity of starch ingested by the host and the size and reproductive rate of *H. diminuta* was demonstrated for a range of from 0.1 to 3.0 grams of starch per day (Read *et al.*, 1958). These data indicate that both quantity and quality of carbohydrate drastically affect the worm.

Only glucose and galactose were found to be metabolized by the worms *in vitro* (Read, 1956; Laurie, 1957). The major acid excretion product was lactic acid (Read, 1956). The production of this acid was enhanced by glucose addition, anaerobic incubation conditions, and starvation (providing the worms were incubated with glucose). Starvation stimulated both metabolism and glycogenesis (Read, 1956).
The rate of glycogenesis during in vitro incubations with glucose was 2.6 times greater in worms starved 48 hours than in worms starved 4 hours when the rate was calculated on a per unit weight basis (Read and Rothman, 1957a). The ratio of glycogen synthesized to glucose utilized rose with starvation indicating more efficient glycogenesis.

The preceding results indicate the importance of carbohydrate for the maintenance and growth of H. diminuta. Synthesis and utilization of glycogen appear to be of crucial importance in the worm's metabolism. This carbohydrate requirement is fairly unique since very few free-living forms show it (Read, 1950).

Campbell (1960a), during studies on pyrimidine metabolism in Hymenolepis diminuta, found that the major end products of the reductive degradation of (2-C^{14})uracil were C^{14}O_{2}, (1-C^{14})alanine, and a (C^{14}) organic acid tentatively identified as succinate. He then suggested that the labelled alanine and succinate could have resulted from CO_{2} fixation.

Fairbairn et al. (1961) observed a great stimulation of glucose utilization and glycogenesis in H. diminuta during incubations in the presence of CO_{2}. These workers also found, in contrast to Read's results, that succinate was the major fermentation product. The CO_{2} stimulation of glycogenesis was confirmed by Kilejian (1963) who also found that the acanthocephalan Moniliformis dubius did not show the same stimulation.
In view of the importance of carbohydrate metabolism in this worm and the indications of the existence of CO₂ fixation and its importance in glycogenesis, a study was made of carbon dioxide fixation in _H. diminuta_ and its relation to glycogenesis.
Materials and Methods

Biological Materials: Young male Holtzman rats were infected with 10 to 30 cysticercoids of Hymenolepis diminuta from either Tenebrio molitor or Tribolium confusum larvae. After killing the rat by a sharp blow on the head, its gut was removed and the worms were expelled by flushing with a syringe-full of Krebs-Ringer solution (Umbreit, Burris and Stauffer, 1964), pH 7.4 with 0.05 M Tris-HCl buffer. The worms were then washed twice in the saline solution and were used either for in vivo incubations or for the preparation of acetone powders.

Preparation of Tissue: Acetone powders were prepared by homogenizing the washed worms in 10 volumes (ml per g) of cold acetone (-20°C) in a Servall Omni-Mixer at high speed for about a minute. The homogenate was then filtered with suction and washed twice with acetone. The residual acetone was removed by sucking air through the powder for a few minutes. It was then dried overnight in vacuo over concentrated sulfuric acid and stored at -20°C until used.

The acetone powders were extracted by homogenizing the powders in glass-distilled water on the basis of 100 mg/ml extraction fluid. If the preparation was to be dialyzed, extraction was with the same buffer used for dialysis. The extracts were centrifuged at 1-2°C for 10-20 minutes at 5,000-20,000xg.
Reagents: Reagents were of analytical grade where possible. Most substrates and cofactors were purchased from either Sigma Chemical Company, St. Louis, Mo., or California Corporation for Biochemical Research, Los Angeles, Cal. NaHCO$_3$ was obtained from the Volk Radiochemical Company, Chicago, Ill.

Analytical Methods: Organic acids were separated either by thin layer chromatography using ethanol: concentrated NH$_3$: water (80:5:15, by vol.) on silica gel G 0.5 mm in thickness (Brinkmann Instruments Inc.) or by chromatography on Whatman No. 1 filter paper using 3-methyl-1-butanol saturated with 4.0 M formic acid (Flavin and Ochoa, 1957).

2,4-Dinitrophenylhydrazones of the keto acids were prepared and purified according to the procedure of El Hawary and Thompson (1953). The dinitrophenylhydrazone of $\alpha$-ketoglutaric acid was separated by thin layer chromatography on silica gel with 2-methyl-2-butanol: ethanol: water (5:1:4, by vol.). The dinitrophenylhydrazones of oxalacetic acid and pyruvic acid were separated with 1-butanol: ethanol: 0.5 N NH$_4$OH (7:1:2, by vol.) on silica gel or Whatman No. 3 filter paper (El Hawary and Thompson, 1953). All three dinitrophenylhydrazones could be separated quite well in one dimension by chromatography with 2-methyl-2-butanol: ethanol: water (5:1:4, by vol.) on Whatman No. 3 filter paper which had been washed with 0.2 M potassium phosphate buffer of pH 6.2 and air-dried overnight (personal communication from
Dr. H. Wager, see Isherwood and Cruickshank, 1954).

Free amino acids were extracted and chromatographed according to Campbell (1960 b) with the exception that the acids from the chloroform extraction were placed on a Dowex-50 (H⁺) column, washed, and eluted with 2N NH₃ water. The eluate was then evaporated to dryness and the acids taken up for spotting in 10% 2-propanol.

Radioactivity measurements were with a Nuclear-Chicago model D47 gas flow detector. Radioautograms were prepared with Ansco non-screen X-ray film.

The protein content of enzyme preparations was determined according to Lowry et al. (1951) using crystalline bovine serum albumin (Sigma) as the standard. A unit of enzyme activity is defined as that amount catalyzing the incorporation of one umole of carbon dioxide or the production of one umole of phosphoenolpyruvate per minute at 38.0°. The specific activity, where given, is in units per mg protein.

Phosphoenolpyruvate was measured by the method of Lohmann and Meyerhof (1934) as described by Bandurski and Lipmann (1956) and modified by Nordlie and Lardy (1963). The $P_i$ formed was determined according to the procedure of Lowry and Lopez (1946).

**Measurement of Enzyme Activity:**

Transaminase activities were determined according to the method of Rowsell (1956) with the exception that the amino acid formed was estimated according to the procedure
of Powden (1951). The assay mixture consisted of 40 μmoles of the amino acid; 40 μmoles of the keto acid; 20 μgrams of pyridoxal phosphate; enzyme; and 50 μmoles of phosphate buffer (KH₂PO₄-Na₂HPO₄), pH 7.4 in a total volume of 2.1 ml.

Phosphoenolpyruvate carboxykinase activity was measured in two ways. The exchange incorporation of C¹⁴O₂ into oxaloacetate was determined according to the procedure of Utter and Kurahashi (1954). The assay mixture contained the enzyme; 2 μmoles MnCl₂; 2 μmoles reduced glutathione; 60 μmoles oxaloacetate; 100 μmoles Tris-HCl (or Tris-acetate) buffer, pH 7.4; and 50 μmoles + 1 μcurie NaHCO₃ in a final volume of 2.0 ml. Phosphoenolpyruvate formation from oxaloacetate was measured according to the assay of Nordlie and Lardy (1963). The assay mixture consisted of 50 μmoles Tris-HCl buffer, pH 7.4; 20 μmoles KF; 2.5 μmoles reduced glutathione; 9.0 μmoles of ATP or ITP; 7.0 μmoles oxaloacetate (Tris salt); 22.4 μmoles MgSO₄; and enzyme. Manganous ion was replaced by magnesium ion in this assay because it causes PEP hydrolysis under the alkaline conditions used for PEP analysis. Fluoride ion was used to inhibit any phosphatase and enolase activities present which could remove both the nucleotide and PEP (Nordlie and Lardy, 1963).

Malic enzyme activity was determined with three different assay procedures. The activity was assayed optically by measuring the rate of TPN reduction in the presence of malate (Ochoa et al., 1948). The assay mixture contained the enzyme;
75 μmoles of Tris-HCl buffer, pH 7.4; 4 μmoles MnCl₂; 0.135 μmole TPN; and 1.5 μmoles L-malate in a final volume of 3.0 ml. Exchange incorporation of C¹⁴O₂ into malate was measured using an assay system consisting of the enzyme; 100 μmoles potassium phosphate buffer, pH 6.0; 2 μmoles MnCl₂; 50 μmoles L-malate; 1 μmole of TPN or DPN; and 50 μmoles of NaHCO₃ containing 1 μcurie C¹⁴. Direct fixation into malate was determined with an assay mixture containing the enzyme; 100 μmoles potassium phosphate buffer, pH 6.0; 2 μmoles MnCl₂; 50 μmoles sodium pyruvate; 1 μmole of TPNH or DPNH; and 50 μmoles of NaHCO₃ containing 1 μcurie C¹⁴. The final volume in both cases was 2.0 ml.

"Malate synthesizing enzyme" activity was determined by measuring the incorporation of C¹⁴O₂ into the acid- and heat-stable fraction in a mixture containing the enzyme; 100 μmoles Tris-acetate buffer, pH 7.4; 6 μmoles MgCl₂; 2 μmoles reduced glutathione; 1 μmole CoA; and 10 μmoles + 1 μcurie NaHCO₃ in a total volume of 2.0 ml. The development of this assay mixture is explained with the results.

The general procedure for enzyme assays, except for TPN-reduction experiments and transaminase assays, was as follows. Aerobic assays were carried out in 25 ml Erlenmeyer flasks. For anaerobic conditions, the incubations were performed in double side-armed Warburg flasks and the flasks were flushed with nitrogen for 3 minutes immediately before the temperature equilibration period. All incubations were carried out
in a constant temperature shaker at 38\(^\circ\). The reactions were started by the addition of enzyme and/or C\(^{14}\)-bicarbonate and were stopped with the addition of either 0.1 ml of 10N H\(_2\)SO\(_4\) or 0.2 ml of 20\% (w/v) trichloroacetic acid. The acid solutions were placed under the hood to remove the residual C\(^{14}\)O\(_2\) and were then centrifuged to remove the precipitated protein. A 0.1 ml or 0.2 ml sample was then plated on a 32 mm diameter stainless steel planchet and either dried in vacuo at 4\(^\circ\) over calcium chloride for the carboxykinase reaction or under an infrared heat lamp for the malic enzyme and "malate synthesizing enzyme" studies. If PEP formation was to be determined, the incubations were stopped with 2.0 ml of 5\% (w/v) trichloroacetic acid, centrifuged at 20,000xg for 15 minutes, and analyzed by the modified method of Lohmann and Meyerhof.

Conditions for individual experiments are given in the legends accompanying the tables and graphs. Any other pertinent information on methods will be given with the results.
Results

In Vivo Studies:

Indications of the existence, nature, extent, and physiological sensitivity of carbon dioxide fixation in *Hymenolepis* were originally obtained through a series of in vivo experiments.

Two grams of 25 day old worms were incubated for one hour with one μcurie NaHCO₃ and 100 mg glucose in 10 ml of the buffered Krebs-Ringer solution. Heat-killed worms were used in the control flask and anaerobic conditions were achieved in one experimental flask by flushing with nitrogen during the half hour preincubation period. The incubation was stopped by the addition of 60 ml of 100% ethanol. The flask contents were homogenized at high speed in a Serval Omni-Mixer. After allowing the homogenate to sit at 4°C overnight, it was centrifuged at 4,000xg for 20 minutes. Amino acids were removed from the supernatant with Bio-Rex 40 (Cal. Biochem.) in the hydrogen form, prepared according to Partridge and Westfall (1949). The organic acids were then extracted and purified according to the procedure of Ballamy (1961) using Amberlite IRA-400 in the carbonate form, prepared as described by Elliott (1954). The resulting eluate was treated with 4 ml of 1% 2,4-dinitrophenylhydrazine in 2N HCl and the dinitrophenylhydrazones were extracted and purified according to El Hawary (1953). These were separated
by thin layer chromatography and were compared with standards. After extraction of the dinitrophenylhydrazones, the aqueous solution containing the remaining acids was evaporated and spotted for thin layer chromatography using the ethanol: NH₃: water system.

Using these methods, a high level of incorporation of C¹⁴O₂ into the organic acid fraction was found (Table 1.). Complete separation of all the suspected organic acids could not be achieved by the thin layer technique. For example, it was impossible to differentiate between fumarate and succinate, and the identity of lactate was also uncertain. The major portion of the C¹⁴ was in the fumarate-succinate fraction. Malate was not detected in the aerobic incubations, but did appear in the anaerobic incubations. No α-ketoglutarate could be found in this or subsequent experiments. A low level of radioactivity was detected in pyruvate under anaerobic conditions. Under these same conditions, no radioactivity was detectable in oxalacetate.

Because the level of radioactivity found in the keto acids was so low as to be almost insignificant, an attempt was made to find more significant indications of C¹⁴ incorporation. Approximately 4 grams of worms were incubated with 10 μcuries NaHCO¹⁴O₃ and 200 mg glucose in 20 ml saline for one-half hour. The experimental flasks were treated as before. The isolated dinitrophenylhydrazone derivatives were separated by chromatography on Whatman No. 3 filter paper with the butanol:ethanol:NH₃ water system.
<table>
<thead>
<tr>
<th>Experimental</th>
<th>cpm in acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OAA Pyruvate &quot;Lactate&quot; Fu.-Su. Malate</td>
</tr>
<tr>
<td>Control</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>Aerobic Incubation</td>
<td>0 7 155 3,366 not present</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0 0 107 2,131 4</td>
</tr>
</tbody>
</table>

TABLE I. Incorporation of $^{14}O_2$ into Organic Acids
Two chromatograms, one each of the aerobic and anaerobic incubation products, were placed on X-ray film for radioautography, while the compounds were eluted from a duplicate set of chromatograms with 1 N NaOH. The alkaline eluates were acidified, and the compounds were extracted with ether. After plating these extracts on aluminum planchets 32 mm in diameter, the incorporated radioactivity was determined.

No radioactivity was detected in the oxalacetate isolated by this method. The pyruvate from 1 gram of worms contained 23 cpm in the aerobic experiment and 18 cpm in the anaerobic experiment. The radioautograms also indicated that more $^{14}C$ was incorporated into pyruvate aerobically than anaerobically. A small amount of radioactivity was incorporated into oxalacetate formed anaerobically, but not into that formed aerobically. Under aerobic conditions, radioactivity was incorporated into an unknown having an Rf value slightly lower than oxalacetate in the chromatography system used.

In view of these results, it was of interest to study the effect of incubation conditions on the incorporation of $^{14}C$ from NaHC$^{14}O_3$ into certain free amino acids. Due to the presence of transaminase activity in most animal tissues, incorporation of $^{14}C$ into keto acids should result in radioactive alanine, aspartate, and glutamate. These compounds are quite stable to isolation procedures in contrast to some of the keto acids and therefore provide a reliable means of
measuring indirectly the incorporation of C\textsuperscript{14} \textit{into} the keto acids (Awapara and Campbell, 1964). Two grams of worms were incubated with 5 \textmu curies NaHC\textsuperscript{14}O\textsubscript{3} and 200 mg glucose in 20 ml saline for 2 hours. Incubations were carried out both aerobically and anaerobically. After stopping the reactions by the addition of 4 volumes of 95% ethanol, the amino acids were isolated, purified, and chromatographed as described by Campbell (1960). Radioautograms were prepared from the chromatograms to detect any incorporated radioactivity.

A visual estimation of chromatograms treated with ninhydrin indicated that much more alanine and proline were present under aerobic than anaerobic conditions. Radioactivity from (C\textsuperscript{14}) bicarbonate was incorporated mainly into alanine and, to a much lesser extent, into aspartate. Enough radioactivity was present in the aspartate to give a slight exposure of the X-ray film. Elution of the aspartate failed to result in detectable radioactivity. Under aerobic conditions, 8170 cpm were incorporated into alanine from 1 g of tissue, and 5300 cpm were incorporated anaerobically.

The fact that aspartate did indeed contain a small quantity of label was demonstrated by isolating and purifying the remaining aspartate in the following manner. The 10% 2-propanol solution of the amino acids resulting from the anaerobic incubation was evaporated to dryness and the residue carefully taken up in 5 ml of water. Ten mg of carrier aspartate was then added. After adjusting the pH of the
solution to 6 (p Hydrion paper), it was boiled and excess copper carbonate was added. The undissolved copper carbonate was filtered off and the solution was cooled overnight at 4°. The precipitated copper salt of aspartic acid was collected and recrystallized twice by redissolving in warm dilute HCl and reprecipitating as the copper salt. The aspartic acid was then regenerated by suspending the copper salt in water and bubbling hydrogen sulfide through the suspension for 30 minutes. The precipitated copper sulfide was filtered off and the filtrate concentrated to about 1 ml with heat. The aspartic acid was precipitated by the addition of sufficient ethanol to make the solution at least 80% ethanol by volume. After allowing the ethanolic solution to stand overnight in the cold, the aspartic acid crystals were centrifuged, washed twice with 100% ethanol, plated and counted. The purified aspartate was found to contain 259 cpm. The identity and purity of the aspartate were confirmed by its decomposition point which was about 275° as determined with a Fisher-Johns melting point apparatus.

Neither in these experiments nor in any experiments carried out in this laboratory has it been possible to detect incorporation of (C^{14}) bicarbonate into α-ketoglutaric acid or glutamic acid, although *Hymenolepis* is known to contain fairly high levels of free glutamic acid (Campbell, 1963). The transaminase activities necessary to account for the conversion of the requisite keto acids to the correspond-
ing amino acids were found to be present and significantly active in *Hymenolepis* as shown in Table 2. The lack of labelled glutamic acid might signify that the presence of glutamic acid in the worm is a consequence of its uptake from the environment as previously suggested by Wertheim et al. (1960). Read (1953) did demonstrate the presence of a pyridine nucleotide-linked L-glutamic dehydrogenase in *Hymenolepis*. It might be that the combination of the transaminase activity involving glutamate and the dehydrogenase activity is so low that any α-ketoglutarate produced is undetectable by the methods employed. Read (1953) was not able to demonstrate the presence of citric or isocitric acid dehydrogenases, and neither citrate nor α-ketoglutarate were found to stimulate respiration (Read, 1956). The present failure to find α-ketoglutarate or the incorporation of C\(^{14}\) into glutamic acid provides further indication that *Hymenolepis* probably does not have a functional citric acid cycle.

Read (1951) presented evidence for the presence of a glycolytic pathway essentially the same as that found in vertebrates. It had also been demonstrated that worms from starved hosts had a higher rate of glycogenesis in the presence of glucose *in vitro* than worms from normal hosts (Daugherty, 1956; Read and Rothman, 1957). Fairbairn *et al.* (1961) found that the presence of bicarbonate in incubation mixtures greatly stimulated glycogenesis in *Hymenolepis*. 
TABLE 2. Transaminase Activities in *Hymenolepis diminuta*

<table>
<thead>
<tr>
<th>Reaction</th>
<th>μMoles product/gram tissue/hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Aspartate + α-Ketoglutarate → Glutamate + Oxalacetate</td>
<td>140.3</td>
</tr>
<tr>
<td>II. Glutamate + Oxalacetate → Aspartate + α-Ketoglutarate</td>
<td>220.1</td>
</tr>
<tr>
<td>III. Alanine + α-Ketoglutarate → Glutamate + Pyruvate</td>
<td>57.1</td>
</tr>
<tr>
<td>IV. Glutamate + Pyruvate → Alanine + α-Ketoglutarate</td>
<td>53.8</td>
</tr>
</tbody>
</table>

Assay: The assay mixture contained 40 μmoles amino acid; 40 μmoles keto acid; 20 μgrams pyridoxal-phosphate; and 50 μmoles phosphate buffer (KH₂PO₄-Na₂HPO₄), pH 7.4, in a total volume of 2.1 ml. Enzyme solution (1.0 ml) was prepared by dialyzing a 10% tissue homogenate against 0.05 M phosphate buffer, pH 7.4 at room temperature for 24 hours. Incubations were carried out under N₂ for 60 minutes at 30°.
Kilejan (1963) reconfirmed Fairbairn's results and found that CO$_2$ did not have the same effect on glycogenesis in *Moniliformis dubius*. Because of the apparent importance of CO$_2$ and the host's nutritional state in the stimulation of glycogenesis in *Hymenolepis*, C$^{14}$O$_2$ incorporation into glycogen was studied.

Approximately three grams of worms were incubated for two hours at $38^\circ$ with 5 μcuries NaHC$^{14}$O$_3$ and 200 mg glucose in 20 ml of saline. Anaerobic conditions were obtained as before and heat-killed worms were again used as controls. The age of the worms in experiment I was 27 days; that of the worms in experiment II, 38 days. The hosts of the starved worms had not been fed for 38 hours prior to the experiment. Incubations were started by (C$^{14}$) bicarbonate addition and stopped by removing the worms from the solution, blotting them, and placing them in 50 ml glass centrifuge tubes containing 20 ml of hot 30% (w/v) KOH. After KOH digestion for one hour in a boiling water bath, the tubes were cooled to 40-50°C and the contents transferred to 100 ml beakers. The glycogen was precipitated by the addition of 36 ml of 95% ethanol to each beaker. It was then centrifuged and redisolved in 10 ml of 10% (w/v) trichloroacetic acid. Any protein present was removed by centrifugation and 20 ml of 95% ethanol were added to the supernatant. After centrifugation, the glycogen was suspended in ethanol, plated, weighed, and counted.
In order to make certain that the label was incorporated into the glucosyl units of the glycogen and not due to contamination, a 20 mg sample from the aerobically incubated starved worms was hydrolyzed in 2 ml of $\text{N}_2\text{H}_2\text{SO}_4$ at 100° C for 6 hours. After centrifuging the hydrolysate and diluting the supernatant to 10 ml with water, 3 ml of phenylhydrazine reagent were added and the solution was heated in a boiling water bath for 45 minutes. The hydrazones were washed five times with water and recrystallized twice from ethanol. The resulting yellow-orange crystals gave the melting point reported for glucosazone (208°C) and contained 340 cpm/mg glucosazone.

As table 3 illustrates, glycogenesis appears to be tremendously sensitive to both the incubation conditions and to the worm's nutritional state. Read and Rothman (1957) obtained a 2.6-fold stimulation of in vitro glycogen synthesis when comparing worms starved 4 hours and those starved 48 hours. According to the present data, there is also a 2.6-fold stimulation of CO$_2$ incorporation when the worms are starved for 38 hours. The incubation conditions are not exactly the same, but the exact correspondence is a good indication that CO$_2$ fixation and glycogen synthesis are probably directly linked.

The occurrence of C$^{14}$O$_2$ label in glycogen, pyruvate, and the dicarboxylic acids argues strongly for the presence of a connecting link between glycolysis and the extant fragment
TABLE 3. *In Vivo* Incorporation of $^{14}\text{C}_2\text{O}_2$ into Glycogen

<table>
<thead>
<tr>
<th>Incubation</th>
<th>μMoles/mg Glycogen</th>
<th>μMoles/g tissue/hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.000</td>
<td>0.00</td>
</tr>
<tr>
<td>Anaerobic (normal)</td>
<td>0.003</td>
<td>0.16</td>
</tr>
<tr>
<td>Aerobic (normal)</td>
<td>0.011</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.000</td>
<td>0.00</td>
</tr>
<tr>
<td>Aerobic (normal)</td>
<td>0.011</td>
<td>0.52</td>
</tr>
<tr>
<td>Aerobic (starved)</td>
<td>0.155</td>
<td>1.36</td>
</tr>
<tr>
<td>Anaerobic (starved)</td>
<td>0.015</td>
<td>0.08</td>
</tr>
</tbody>
</table>
of the citric acid cycle. The lowering of the incorporation of \( ^{14}C \)O\(_2\) into pyruvate and alanine, the accumulation of malate, and the appearance of radioactivity in oxalacetate during anaerobic incubation suggests that the link is energy dependent and might involve oxalacetate. Finally, if a net synthesis of glycogen and a stimulation of metabolism is to occur in the presence of CO\(_2\), there must be a CO\(_2\) fixation reaction independent of the postulated connecting link. Preferably this would result in the synthesis of one of the dicarboxylic acids of the citric acid cycle fragment. In this case, as Read (1950) has suggested, the net synthesis of acids could furnish substrate for both glycolysis and terminal electron transport.
In Vitro Studies:

Because of the results obtained in vivo, the next portion of this study consisted of a systematic search for the enzymes involved in CO₂ fixation in Hymenolepis.

Phosphoenolpyruvate Carboxykinase:

Phosphoenolpyruvate carboxykinase was first demonstrated in avian liver by Utter and Wood (1946). The mechanism was proposed to be the following as the result of a series of studies (Utter and Kurahashi, 1954; Utter, Kurahashi and Rose, 1954; Kurahashi, Pennington and Utter, 1957).

\[
\text{Mn}^{+2} \quad \text{CO}_2 + \text{phosphoenolpyruvate} + \text{GDP} \rightarrow \text{oxalacetate} + \text{GTP}
\]

The in vivo studies had indicated the presence of an energy dependent connecting link between glycolysis and the dicarboxylic acids, possibly involving oxalacetate. The carboxykinase seemed a good candidate for this link since it was freely reversible and energy requiring. The phosphoenolpyruvate produced could contribute to glycogenesis via the glycolytic pathway, of which it is a member.

The assay of dialyzed acetone powder extracts of Hymenolepis according to the procedure of Utter (1954) indicated the presence of a quite active phosphoenolpyruvate carboxykinase (Table 4). A reduced glutathione requirement could not be demonstrated. However this requirement is only shown after purification of the enzyme (Utter, Kurahashi and Rose, 1954) and not in crude preparations such as used here. Similarly, a complete \( \text{Mn}^{+2} \) dependence was not shown in this
### TABLE 4. Phosphoenolpyruvate Carboxykinase Assay

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles CO₂ incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.22</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.15</td>
</tr>
<tr>
<td>+ITP</td>
<td>7.79</td>
</tr>
<tr>
<td>+ATP</td>
<td>2.82</td>
</tr>
<tr>
<td>-MnCl₂ + ITP</td>
<td>3.08</td>
</tr>
<tr>
<td>- GSH</td>
<td>7.36</td>
</tr>
</tbody>
</table>

**Assay:** The complete system contained the enzyme; 2 μmoles MnCl₂; 2 μmoles GSH; 60 μmoles oxalacetate; 100 μmoles Tris-HCl buffer, pH 7.4; and 50 μmoles + 1 μcurie NaHCO₃ in a final volume of 2.0 ml. The oxalacetate was neutralized with 0.5 M Na₃PO₄. Two μmoles of either ATP or ITP were added where indicated. The acetone powder extract was dialyzed overnight against two one-liter changes of 10⁻³ M potassium phosphate buffer, pH 7.8. Enzyme solution containing 3.4 mg protein was added to each flask. Incubations were carried out for 15 minutes under anaerobic conditions.
experiment. Apparently all the manganese was not removed by dialysis, as was indicated by a later experiment where EDTA was added. This preliminary experiment also indicated that the activity had the same nucleotide requirements as reported for the avian enzyme.

In order to demonstrate the presence of the activity conclusively, it was felt necessary to identify the products. Further incubations were run under the same conditions which resulted in the exchange incorporation of 58,770 and 55,522 cpm. Dinitrophenylhydrazones from the most active sample were extracted and purified as before. They were then separated along with added carrier dinitrophenylhydrazones by chromatography on Whatman No. 3 filter paper using the 2-methyl-2-butanol:ethanol:water system. After elution with 0.1 M glycine-NaOH buffer, pH 8.4, the solutions were acidified and the hydrazones extracted with ether. Upon plating and counting, essentially all the activity recovered (591 cpm) was found in oxalacetate. When the other sample was treated in the same manner and the activity followed through each step, a similar loss was observed. Despite the large loss of radioactivity, the experiment still provided an indication that oxalacetate was at least one of the fixation products.

More conclusive evidence for the participation of oxalacetate was obtained by incubations with and without oxalacetate followed by aluminum decarboxylation of the oxal-
acetate in the reaction mixtures. This specific aluminum decarboxylation of oxalacetate was used by Utter and Kurahashi (1954) to demonstrate $^{14}\text{C}_2$ incorporation into oxalacetate. The decarboxylation was accomplished by placing the reaction mixtures in Warburg flasks with CO$_2$-free 3.5M NaOH in the center-wells. After adding 0.1 ml of 33% AlCl$_3$ to each flask, the flasks were incubated with shaking at 38°C for one hour. The mixtures were then plated and counted again.

Almost all activity was lost upon oxalacetate deletion, and most of the radioactivity disappeared with decarboxylation (Table 5). The remaining radioactivity could have been the result of randomization of the C$^{14}$ after fixation into oxalacetate. The preceding results demonstrated that oxalacetate was indeed the fixation product.

The reversibility of the reaction was demonstrated by the measurement of phosphoenolpyruvate formation from oxalacetate in the presence of the necessary cofactors (Nordlie and Lardy, 1963).

Phosphoenolpyruvate was determined by the modified procedure of Lohmann and Meyerhof (1934). After stopping the reaction with the addition of 2.0 ml of 5% (w/v) trichloroacetic acid, the protein was centrifuged at 20,000xg for 15 minutes. The supernatant was neutralized with KOH and then made alkaline by the addition of 0.1 ml of 2N KOH. Two standard solutions of phosphoenolpyruvate were treated
TABLE 5. Oxalacetate Deletion and Decarboxylation Studies

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles CO₂ incorp.</th>
<th>μMoles after Al⁺³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Complete System</td>
<td>6.85</td>
<td>0.58</td>
</tr>
<tr>
<td>- Oxalacetate</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

Assay: The complete system was the same as given in Table 4 with ITP added as the nucleotide. Incubations were carried out anaerobically for 10 minutes using undialyzed acetone powder homogenate containing 5.5 mg protein in each flask.
in the same way in order to determine the percentage of recovery of the analysis. After the addition of 0.3 ml of 0.1 M \textit{I}_2 in KI to each of the experimental samples and one of the phosphoenolpyruvate standards, all the mixtures were allowed to sit for 20 minutes at room temperature. Phosphoenolpyruvate hydrolysis was stopped with the addition of 1 ml of 1 N HCl, and the excess iodine was discharged with sodium bisulfite. The samples were then brought to pH 4 with 0.1 N sodium acetate and were analyzed for P_\text{i} according to the method of Lowry and Lopez (1946). Recovery was determined by subtracting the amount of P_\text{i} found in the phosphoenolpyruvate standard not treated with iodine from that found in the standard subjected to iodine hydrolysis. Recoveries averaged approximately 75% in all analyses. In calculating the amount of phosphoenolpyruvate formed, the amount of P_\text{i} found in the heat killed control was subtracted from that found in the experimental samples.

Phosphoenolpyruvate formation was demonstrated and shown to be responsive to the nucleotide present in the same manner as C^{14}O_2 exchange incorporation (Table 6). This demonstration of the reversal of the reaction, together with the exchange incorporation results, fairly conclusively proved the existence of phosphoenolpyruvate carboxykinase in acetone powder extracts of \textit{Hymenolepis}. Furthermore, the rate of phosphoenolpyruvate formation was great enough to account for the \textit{in vivo} results.
It had been previously demonstrated that ATP had no direct effect upon carboxykinase activity and that it probably acted through ITP or GTP contamination via the action of Berg and Joklik's nucleoside diphosphokinase (Utter et al., 1954; Kurahashi et al., 1957). There were also indications that GTP rather than ITP was the physiologically active phosphate donor (Kurahashi et al., 1957). It therefore seemed necessary to determine the effectiveness of all three of the nucleotides.

As is shown in table 7, ITP gave slightly more stimulation than GTP, and both showed much greater activity than ATP. Kurahashi (1957) also found that ITP would stimulate more than GTP at higher levels when using purified chicken liver enzyme. Only at much lower levels of nucleotide, around 0.01 μmoles, did he find greater GTP stimulation.

The activity also possessed a normal sulfhydryl dependence when the powder extract was incubated with $10^{-3}$ M p-chloromercuribenzoate (Table 8). Inhibition was not overcome by the addition of 15 times as much reduced glutathione. This could have been due to the very high concentration of the inhibitor used. Utter (1954) found a partial reversal in the inhibition of the chicken liver enzyme by using a lower concentration of inhibitor and a higher amount of reduced glutathione. Because of the high amount of activity remaining after deletion of MnCl$_2$ in the first experiment, MnCl$_2$ was again deleted. This time however one μmole of
### TABLE 6. Phosphoenolpyruvate Formation from Oxalacetate

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles phosphate formed</th>
<th>units of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System + ITP</td>
<td>2.27</td>
<td>0.23</td>
</tr>
<tr>
<td>Complete System + ATP</td>
<td>0.41</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Assay: The reaction mixture consisted of 50 μmoles Tris-HCl buffer, pH 7.4; 20 μmoles KF; 2.5 μmoles GSH; 9.0 μmoles of either ATP or ITP; 7.0 μmoles oxalacetate (Tris salt); 22.4 μmoles MgSO₄; and enzyme solution containing 6.5 mg protein in a total volume of 2.0 ml. Incubations were carried out anaerobically for 10 minutes. The enzyme solution was dialyzed overnight against two one-liter changes of 10⁻³ M Tris-acetate buffer, pH 7.4, before use.

### TABLE 7. Nucleotide Requirements of PEP Carboxykinase

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles CO₂ incorp.</th>
<th>units of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>+ATP</td>
<td>0.57</td>
<td>0.06</td>
</tr>
<tr>
<td>+ITP</td>
<td>2.83</td>
<td>0.28</td>
</tr>
<tr>
<td>+GTP</td>
<td>2.52</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Assay: The complete system was the same as given in Table 4. Two μmoles of the required nucleotide were added. Incubations were carried out aerobically for 10 minutes using enzyme solution containing 4.7 mg protein in each flask.
TABLE 8. Cation & Sulphydryl Dependence of PEP Carboxykinase

<table>
<thead>
<tr>
<th>Experimental</th>
<th>µMoles CO₂ incorp.</th>
<th>units of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td>Complete System</td>
<td>2.16</td>
<td>0.17</td>
</tr>
<tr>
<td>-MnCl₂ + EDTA</td>
<td>0.39</td>
<td>0.03</td>
</tr>
<tr>
<td>+ p-CMB</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>+ p-CMB + GSH</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Assay: The complete system was the same as given in Table 4. The enzyme was allowed to sit with the inhibitor for 5 minutes before either the incubation was started or the GSH added. Undialyzed acetone powder extract containing 6.5 mg protein was added to each flask. Incubations were carried out anaerobically for 10 minutes.
EDTA was added to chelate any endogenous ion. Under these conditions the activity did show a more complete ion dependence indicating that the activity in the first experiment was due to undialyzable manganous and/or magnesium ions.

The dependence of the carboxykinase reaction on enzyme concentration and incubation time is shown in Figures 1 and 2. Magnesium ion was found to be equivalent to manganous ion in activating the enzyme and was used whenever phosphoenolpyruvate formation was assayed since manganous ion would cause the hydrolysis of the phosphoenolpyruvate in alkaline solution (Nordlie and Lardy, 1963). The original assay conditions described by Utter (1954) called for anaerobic incubation, but aerobic incubation was found here to give essentially the same results.

The optimum pH for the exchange incorporation of \( ^{14} \text{CO}_2 \) was around pH 6, and the curve greatly resembled the pH curve for the ATP-stimulated decarboxylation of oxalacetate catalyzed by the chicken liver enzyme (Utter et al., 1954). This was quite different from the pH optimum of 8 found by Nordlie and Lardy (1963) for the guinea pig enzyme.

In summary, *Hymenolepis diminuta* has a quite active and readily reversible phosphoenolpyruvate carboxykinase which probably serves as the connecting link between glycolysis and the remnant of the citric acid cycle. The rate of phosphoenolpyruvate formation is great enough to account for the observed *in vivo* incorporation of \( \text{CO}_2 \) into glycogen.
FIGURE 1. PEP Carboxykinase Concentration Dependence

The reaction mixture was the same as given in Table 4. Undialyzed enzyme solution containing 1.4 mg protein/0.1 ml was used. Incubations were for 10 minutes.

O = Anaerobic Incubation

• = Aerobic Incubation

FIGURE 2. PEP Carboxykinase Time Course and Ion Requirement

The reaction mixture was the same as given in Table 4 with 2 µmoles of either MnCl₂ or MgCl₂ being added where indicated. Dialysis was carried out overnight against a liter of 10⁻³ M potassium phosphate buffer, pH 7.8 and enzyme solution containing 7.3 mg protein was added to each flask. Incubations were carried out anaerobically for 15 minutes.

• = + MnCl₂

○ = + MgCl₂

▲ = - Cations
FIGURE 3. pH Curve for $^{14}O_2$ Exchange Incorporation into Oxalacetate

The reaction mixture was the same as given in Table 4 with the exception that 200 μmoles of one of the three indicated buffers at the desired pH was used. The initial pH of the reaction mixture was taken as the correct incubation pH since there was a slight drifting of pH during the reaction. The oxalacetate solutions were brought to the proper pH with Na$_3$PO$_4$. Incubations were carried out aerobically for 10 minutes. Two separate experiments were run and the results were normalized. In the first experiment (Tris-maleate buffers), undialyzed enzyme solution containing 6.6 mg protein was used per flask; in the second experiment, solution containing 6.1 mg protein was used in each flask.

△ = Tris - Acetate Buffer
○ = potassium Phosphate Buffer
● = Tris - Maleate Buffer
Malic Enzyme:

The malic enzyme was first demonstrated in pigeon liver by Ochoa et al. (1948) and subsequently studied in considerable detail by Viega Salles et al. (1950). The enzyme catalyzes the fixation of CO$_2$ into malate according to the following reaction.

\[
\text{CO}_2 + \text{pyruvate} + \text{TPNH} \xrightleftharpoons{\text{Mn}^{+2}} \text{L-malate} + \text{TPN}^+ 
\]

Due to the fact that this enzyme is so ubiquitous (see Krebs and Lowenstein, 1960) and would provide the necessary dicarboxylic acid substrate for net glycogen synthesis, acetone powder extracts of *Hymenolepis* were assayed for the activity by measuring the rate of TPN reduction in the presence of malate (Ochoa et al., 1948) on a Beckman DB recording spectrophotometer. No significant malic enzyme activity was found by this method, although chicken liver acetone powder gave good activity. Addition of *Hymenolepis* extracts to the chicken liver enzyme did not cause inhibition.

Because Read (see Read and Simmons, 1963), in unpublished work, had found a low level of TPN-dependent malate decarboxylation by manometric measurement, the activity was then searched for through the measurement of the exchange incorporation of C$^{14}$O$_2$ into malate. A very low level of activity was found using this more sensitive technique (Table 9). The activity had both a TPN preference and a requirement for L-malate and manganous ion.
<table>
<thead>
<tr>
<th>Experimental</th>
<th>µMoles CO₂ incorp.</th>
<th>units of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>+TPN</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>+DPN</td>
<td>0.04</td>
<td>0.004</td>
</tr>
<tr>
<td>+DPN - Malate</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>+DPN - MnCl₂</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Assay: The complete system contained 100 µmoles potassium phosphate buffer, pH 6.0; 2 µmoles MnCl₂; 50 µmoles L-malate; 50 µmoles NaHCO₃ containing 1 µcurie C¹⁴; and enzyme in a final volume of 2.0 ml. One µmole of either TPN or DPN were added where indicated. The acetone powder extract was dialyzed overnight against two one-liter changes of 5x10⁻² M Tris-HCl buffer, pH 7.4, and enzyme solution containing 6.0 mg protein was used per flask. Incubations were carried aerobically for 10 minutes.
The reason for the lack of results with the optical assay was clearly due to a combination of the very low level of activity and the necessity of using less protein than in the isotopic assay in order to avoid too much turbidity.

Because of the presence of this activity, it became critical to separate and distinguish it from the PEP carboxykinase reaction. This was done by incubating a dialyzed system capable of fixation by both mechanisms with the three different cofactors and showing differential incorporation into malate and oxalacetate. Incorporation into malate was indicated by the radioactivity remaining after aluminum de-carboxylation of the oxalacetate present. The two activities were found to be completely separate as ATP did not stimulate fixation into malate, and the nicotinamide nucleotides did not stimulate fixation into oxalacetate (Table 10). The TPN-stimulated incorporation compared well with that obtained in the first experiment. However the DPN-stimulated incorporation was much higher, most probably due to its stimulation of malic dehydrogenase which would convert some of the oxalacetate labelled in the absence of added ATP to malate. Apparently dialysis did not remove all the ATP. This resulted in a small background activity.

Direct fixation of CO₂ into malate was also demonstrated (Figure 4). TPNH-stimulated incorporation went up with time and then rapidly fell off. This rapid fall was probably due to the lack of a TPNH-regenerating system and
### TABLE 10. Separation of the Carboxykinase & Malic Enzyme Activities

<table>
<thead>
<tr>
<th>Experimental</th>
<th>µMoles before Al&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>µMoles after Al&lt;sup&gt;3+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete System (10 min.)</td>
<td>0.30</td>
<td>0.00</td>
</tr>
<tr>
<td>+ATP (5 min.)</td>
<td>1.12</td>
<td>0.00</td>
</tr>
<tr>
<td>+ATP (10 min.)</td>
<td>1.72</td>
<td>0.00</td>
</tr>
<tr>
<td>+TPN (5 min.)</td>
<td>0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>+TPN (10 min.)</td>
<td>0.51</td>
<td>0.21</td>
</tr>
<tr>
<td>+DPN (5 min.)</td>
<td>0.17</td>
<td>0.04</td>
</tr>
<tr>
<td>+DPN (10 min.)</td>
<td>0.55</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Assay: The complete system was the same as given in Table 9 with the exception that 50 µmoles of oxalacetate were also added. Two µmoles of ATP and one µmole of TPN or DPN were used as indicated. The enzyme solution was prepared as given in Table 9, and an amount containing 0.4 mg protein was added to each flask. Incubations were carried out aerobically for the durations indicated in the parentheses.
FIGURE 4. Direct Fixation into L-Malate

Assay: The reaction mixture was the same as given in Table 9 with the exception that 50 µmoles of sodium pyruvate were substituted for the malate. One µmole of TPNH, DPNH, and CoA were used where indicated. The dialyzed enzyme was prepared as given in Table 9, and enzyme solution containing 9.6 mg protein was used per flask. Incubations were run aerobically for the times indicated. Incubations to determine CoA effects were for 10 minutes.

○ = + TPNH

● = + DPNH

□ = - TPNH, DPNH, or Pyruvate

△ = + Heat Killed Enzyme
the subsequent decarboxylation of the malate formed. When exchange incorporation into malate was being measured, the large excess of malate could serve as a substrate for TPNH production via the reverse of the fixation reaction. The previous experiment had shown that a dialyzed extract would fix CO$_2$ in the presence of oxalacetate even without the addition of ATP; presumably due to endogenous, undialyzed nucleotide. The continued rise of DPNH-stimulated incorporation may have been due to stimulation of malic dehydrogenase. This would provide some oxalacetate for fixation and then distribute the resulting label into malate. Even so, some decrease in the rate of fixation was observed after the first 5 or 10 minutes. No activity was observed upon the deletion of either the nicotinamide nucleotides or pyruvate. Coenzyme A had a slight inhibitory effect upon both the TPNH- and the DPNH-stimulated incorporations. The import of this observation will be discussed later in the proper context.

*H. diminuta* has what appears to be a very low level of malic enzyme activity. It probably does make some contribution to the dicarboxylic acid pool, but seems to be much less active than either PEP carboxykinase or "malate synthesizing enzyme" activity. This is assuming that the optical and the isotope assays were somewhere near the pH optima. The malic enzyme has two separate pH optima as was mentioned previously, and these may vary from animal to animal. Therefore these experiments can serve only as a crude indication of activity.
"Malate Synthesizing Enzyme":

Because the other major route of carbon dioxide fixation in animal tissues is into succinate via propionyl carboxylase, a preliminary assay for the presence of the activity in undialyzed acetone powder homogenate was carried out according to the procedure of Tietz and Ochoa (1959). Activity was detected at pH 7.4. However when the same assay was repeated at pH 8.0, the pH optimum for pig heart propionyl carboxylase, no activity was found. Repetition of the assay at pH 7.4 again resulted in label incorporation. A sample from this assay with 24,721 cpm incorporation was extracted with ether and chromatographed with added carriers and standards using 3-methyl-1-butanol saturated with 4.0 M formic acid. Most of the recovered activity was found in malate indicating that it might be the fixation product instead of succinate. (Table 11).

Dialysis of the powder extract overnight against two one-liter changes of 10^{-3} M Tris-HCl buffer, pH 7.4, resulted in a complete loss of activity. Repetition of dialysis overnight in ice-cold 0.02 M Tris-acetate buffer containing 10^{-3} M reduced glutathione and 10^{-3} M EDTA for protection also resulted in complete loss of activity. It appeared that required cofactors and/or substrates were being lost upon dialysis and not replaced in the enzyme assay.

At this point a deletion experiment was run to determine what components of the assay mixture were necessary.
<table>
<thead>
<tr>
<th>Acid</th>
<th>cpm incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinic Acid</td>
<td>566</td>
</tr>
<tr>
<td>Fumaric Acid</td>
<td>276</td>
</tr>
<tr>
<td>Malic Acid</td>
<td>1,810</td>
</tr>
</tbody>
</table>

Assay: The reaction mixture contained 1 µmole CoA; 1 µmole propionate; 3 µmoles ATP; 6 µmoles MgCl₂; 2 µmoles GSH; 100 µmoles Tris-acetate buffer, pH 7.4; 10 µmoles and 1 µcurie NaHCO₃; and enzyme in a final volume of 2.0 ml. Undialyzed acetone powder homogenate containing 5.5 mg protein was added to each flask. Incubations were carried out aerobically for 15 minutes.
The undialyzed acetone powder demonstrated only magnesium and coenzyme A requirements (Table 12).

Flavin and Ochoa (1957) used malonate to inhibit succinic dehydrogenase thus allowing C\textsuperscript{14} from NaHCO\textsubscript{3} to accumulate in methylmalonate and succinate. Malonate inhibition was tried in order to ascertain the nature of the fixation product. At the same time other deletions were made from the complete system in order to determine optimal assay conditions for further studies.

The data indicated, contrary to the preceding experiment, that ATP had an inhibitory effect on the reaction (Table 13). The highest activity was obtained when all cofactors and substrates except for NaHCO\textsubscript{3} were deleted. There seemed to be enough endogenous material to support the reaction. Malonate was found to completely inhibit the incorporation of CO\textsubscript{2}. From these and previous results it was obvious that the activity was not due to propionyl carboxylase.

A more detailed experiment was then carried out in order to confirm the previous results and to determine other requirements. From this experiment on, the complete system contained enzyme, reduced glutathione, magnesium, buffer, coenzyme A, and sodium bicarbonate. This reaction mixture seemed to give the closest approximation to optimal conditions. As the results in Table 14 indicate, inhibition resulted from the addition of ATP, ITP, GTP, propionate,
### TABLE 12. Assay Mixture Deletion Experiment

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles CO₂ incorp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.68</td>
</tr>
<tr>
<td>-MgCl₂ + 1 μmole EDTA</td>
<td>0.01</td>
</tr>
<tr>
<td>-ATP</td>
<td>0.68</td>
</tr>
<tr>
<td>-GSH</td>
<td>0.67</td>
</tr>
<tr>
<td>-Propionate</td>
<td>0.67</td>
</tr>
<tr>
<td>-CoA</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Assay: The complete system was the same as given in Table 11. Acetone powder homogenate containing 11.2 mg protein was used per flask. Incubation was for 15 minutes.

### TABLE 13. Malonate Inhibition of Activity & Assay Composition

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles CO₂ incorp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.08</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>0.00</td>
</tr>
<tr>
<td>- Propionate &amp; CoA</td>
<td>0.01</td>
</tr>
<tr>
<td>- Propionate, CoA &amp; ATP</td>
<td>0.32</td>
</tr>
<tr>
<td>Buffer + HCO₃ + enzyme</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Assay: The complete system was as given in Table 11. 20 μmoles of malonate were added where indicated. Undialyzed acetone powder extract containing 5.7 mg protein was used per flask. Incubation was for 15 minutes.
TABLE 14. Substrate and Cofactor Requirements

<table>
<thead>
<tr>
<th>Experimental</th>
<th>( \mu \text{Moles CO}_2 ) incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.23</td>
</tr>
<tr>
<td>+ MnCl(_2)</td>
<td>0.17</td>
</tr>
<tr>
<td>+ ATP</td>
<td>0.11</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>0.01</td>
</tr>
<tr>
<td>+ Propionate</td>
<td>0.10</td>
</tr>
<tr>
<td>+ Propionate &amp; ATP</td>
<td>0.10</td>
</tr>
<tr>
<td>Complete System - CoA</td>
<td>0.16</td>
</tr>
<tr>
<td>+ ATP</td>
<td>0.13</td>
</tr>
<tr>
<td>+ ADP</td>
<td>0.16</td>
</tr>
<tr>
<td>+ ITP</td>
<td>0.08</td>
</tr>
<tr>
<td>+ GTP</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Assay: The complete system consisted of 100 \( \mu \)moles Tris-acetate buffer, pH 7.4; 6 \( \mu \)moles MgCl\(_2\); 2 \( \mu \)moles GSH; 1 \( \mu \)mole CoA; 10 \( \mu \)moles and 1 \( \mu \)curie NaHCO\(_3\); and enzyme in a total volume of 2.0 ml. Other additions where indicated were 3 \( \mu \)moles of the nucleotides; 6 \( \mu \)moles MnCl\(_2\); 20 \( \mu \)moles malonate; and 5 \( \mu \)moles propionate. Undialyzed acetone powder homogenate containing 14.6 mg protein was added to each flask and the incubations were carried out for 15 minutes.
and malonate thus confirming the preceding experiment. Coenzyme A again stimulated CO$_2$ incorporation. Manganous ion was found to activate the enzyme, but not as effectively as magnesium. The observed nucleotide inhibition of CO$_2$ incorporation separated the activity from PEP carboxykinase, pyruvic carboxylase, and other fixation reactions using these nucleotides as an energy source.

The activity did show a normal dependence on enzyme concentration and incubation time (Figures 5 and 6). The rate of incorporation decreased with increased incubation time, due perhaps to exhaustion of unsupplied substrates. It had previously been found that CoA did not stimulate malic enzyme activity, but rather gave a slight inhibition. Further evidence that the activity was not due to the malic enzyme was provided by the observation that both TPNH and DPNH inhibited CO$_2$ incorporation in this experiment. However malate addition did result in a 4-fold stimulation of incorporation, thus confirming earlier chromatographic indications that malate might be the fixation product.

In order to demonstrate a selective stimulation by malate, incubations were run with malate and the other dicarboxylic acids. Pyruvate and glyoxylate were also tried. Glyoxylate was included because it was felt that the worm might have an enzyme similar to or identical with malate synthetase which produces malate from glyoxylate and acetyl CoA.
FIGURE 5. "Malate Synthesizing Enzyme" Concentration Dependence

The incubation mixture was the same as in Table 14. Undialyzed acetone powder extract containing 1.6 mg protein per 0.1 ml was used. All incubations were carried out for 10 minutes.

FIGURE 6. "Malate Synthesizing Enzyme" Time Course

The incubation conditions were the same as in Figure 5. Undialyzed acetone powder extract containing 6.9 mg protein was used in each flask.
TABLE 15. Product and Substrate Requirements

<table>
<thead>
<tr>
<th>Experimental</th>
<th>μMoles CO₂ incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Kill</td>
<td>0.00</td>
</tr>
<tr>
<td>Complete System</td>
<td>0.17</td>
</tr>
<tr>
<td>+ Malate</td>
<td>0.25</td>
</tr>
<tr>
<td>+ Succinate</td>
<td>0.12</td>
</tr>
<tr>
<td>+ Fumarate</td>
<td>0.11</td>
</tr>
<tr>
<td>+ Oxalacetate</td>
<td>0.24</td>
</tr>
<tr>
<td>+ Pyruvate</td>
<td>0.07</td>
</tr>
<tr>
<td>+ Glyoxylate</td>
<td>0.12</td>
</tr>
<tr>
<td>+ ATP</td>
<td>0.03</td>
</tr>
<tr>
<td>+ ADP</td>
<td>0.07</td>
</tr>
<tr>
<td>+ FAD</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Assay: The complete system was the same as given in Table 14 with the exception that 0.5 μmoles of CoA were used per flask. Five μmoles of the acids; 3 μmoles of ATP and ADP; and 1 μmole of FAD were added as indicated. Acetone powder extract containing 7.4 mg protein was added to each flask. Incubation was for 10 minutes.
Only malate and oxalacetate stimulated incorporation (Table 15). Oxalacetate could not be the fixation product since the reaction mixtures from one or two of the experiments had been treated with AlCl₃ with no resulting loss of radioactivity. Apparently the oxalacetate was acting through the mediation of malic dehydrogenase. Neither pyruvate nor glyoxylate seemed to be able to serve as a substrate. All three nucleotides inhibited the incorporation. ADP inhibition was unexpected because of its lack of effect when previously tested.

The previous experiments indicate that the enzyme is real and quite active enough to be physiologically important. It is impossible to estimate exactly how active it really is because of the lack of knowledge of all substrate and cofactor requirements. It does show a cation and coenzyme A requirement. Fixation via this reaction results in malate formation. The activity appears to be different from any other yet found and deserves further investigation.
Physiological Studies:

Earlier experiments on incorporation of $^{14}\text{CO}_2$ into glycogen had indicated a great sensitivity of the incorporation rate to the nutritional state of the worm. It was of interest then to determine whether the enzyme activities for carbon dioxide fixation were also sensitive to starvation.

Rats with 93 day old worms were separated into two groups. One group was starved for 24 hours while the control group was allowed to feed ad libitum. At the end of 24 hours the rats were sacrificed; the worms were removed and acetone powders prepared in the normal manner. These powders were immediately assayed for the carboxykinase activity by measurement of phosphoenolpyruvate formation and for the "malate synthesizing enzyme" activity by measurement of CO$_2$ incorporation under the modified "optimal" conditions. Boiled powders were used as controls.

Phosphoenolpyruvate carboxykinase was found to be very sensitive to nutritional state as shown by the nearly two-fold increase of specific activity as a result of 24 hour starvation (Table 16). The unknown activity, in contrast, showed a slight decline upon starvation. However the activity, even at suboptimal conditions, was active enough to account for the in vivo results. For example, Fairbairn (1961) found that a 14 day-old average 12-worm sample had 0.425 g of protein. Taking an average specific activity of $0.15 \times 10^{-2}$ μmoles/min./mg, an average worm
### TABLE 16. Enzyme Activity in Starved Worms

<table>
<thead>
<tr>
<th>Experimental</th>
<th>Specific Activity ($x10^2$)</th>
<th>PEP Carboxykinase</th>
<th>&quot;Malate Synthesizing Enz.&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Normal Worms</td>
<td></td>
<td>1.34</td>
<td>0.18</td>
</tr>
<tr>
<td>Starved Worms</td>
<td></td>
<td>2.46</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**Assay:** The carboxykinase assay was carried out under the same conditions and with acetone powder homogenate dialyzed in the same manner as given in Table 6. Incubation was for 10 minutes.

The "malate synthesizing enzyme" assay was carried out with undialyzed acetone powder homogenate according to the conditions given in Table 14. Incubation was for 10 minutes.
would be able to fix around 2.2 μmoles per hour. This would be more than enough to account for the in vivo results without taking the malic enzyme activity into account. The slight decrease in "Malate Synthesizing Enzyme" activity upon starvation may have simply been due to the decline in a substrate or necessary cofactor upon starvation. Since all the cofactors and substrates are not supplied in the assay mixture, the enzyme would appear to decline in activity.

A fairly good correlation between PEP carboxykinase specific activity and worm age was observed. The activity increased about 10-fold up to about the age of 200 days and then began to decline as the worms became older. Since the worm shows no signs of senility when kept established in young rats, this decline in activity after a certain age may be due to changes in the rat host rather than in the worm. (Read, personal communication). If given enough time, the host will eventually expel the parasite. Perhaps this decline in activity is a symptom of the expulsion process. This problem should be investigated more thoroughly. Only enough data was obtained in this study to give a broad, qualitative indication that there probably is a connection between the specific activity of the carboxykinase and the worm's age. The rat itself also has both a liver PEP carboxykinase and malic enzyme. The carboxykinase is sensitive to starvation and appears to be linked to carbohydrate synthesis, but the malic enzyme is not abun-
dent enough to account for carbohydrate synthesis (Shrago et al., 1963).

From these results, it appears that PEP carboxykinase may serve as the physiologically sensitive control mechanism or valve which feeds the extra carbon produced by fixation into the Embden-Meyerhof sequence. At the same time, the extra malic acid would also provide a substrate for terminal electron transport via malic dehydrogenase. At the moment, the true activity of the "malate synthesizing enzyme" and its sensitivity to starvation cannot be determined with certainty due to the fact that all its requirements are not supplied in the assay mixture and its pH optimum is not known. However, the general sequence pictured in Figure 7 would account for the experimental observations.
General Discussion

Previous studies have indicated the importance of carbohydrate to the growth and reproduction of *Hymenolepis diminuta* (See Read, 1959). Also the central role of CO\(_2\) in the metabolism of this worm has been demonstrated previously and in the present work. The extremely close correspondence between the stimulation of glycogenesis and CO\(_2\) incorporation into glycogen by starvation supports the intimate connection between fixation and glycogenesis. Read (1956) found that pyruvate failed to stimulate respiration in *Hymenolepis*. This would argue both for the absence of a citric acid cycle and for the irreversibility of the pyruvic kinase reaction. On the other hand, Daugherty (1956) found that pyruvate supported glycogenesis, and that starvation could stimulate this pyruvate-supported glycogenesis. The support of glycogenesis by pyruvate may well be due to fixation resulting in a C\(_4\)-dicarboxylic acid with subsequent channeling of the extra carbon into glycogen by a reversal of the glycolytic pathway from phosphoenolpyruvate. This general route has a good precedent in vertebrate glycogenesis. The fixation could have resulted in malate via the action of the malic enzyme. The present studies indicate that this activity is too low to be of great significance. However as pointed out before, the malic enzyme may be considerably more active *in vivo* due to the fact that the assays may not have been run under optimal conditions. It is
also possible that the pyruvate could have been converted to
the substrate necessary for the "Malate Synthesizing Enzyme"
and then resulted in malate formation. Preliminary studies
have not demonstrated the exact nature of this intermediate
so that it is impossible to ascertain at the moment whether
this is actually the case. Of course there might also be
other fixation mechanisms present which have not yet been
detected.

The fact that there appears to be a lessening of overall
metabolic activity and glycogenesis under anaerobic condi-
tions would imply the lack of high-energy phosphate syn-
thesis under these conditions. This would at least provide
indirect evidence for the absence of the anaerobic phospho-
rylating mechanism found in Ascaris muscle. Furthermore,
unlike the case with Ascaris, a cytochrome oxidase has been
found in Hymenolepis. A direct attempt should be made to
demonstrate either the presence or absence of this anaero-
bic phosphorylating mechanism in Hymenolepis.

Schwade, in unpublished observations, demonstrated
glyconeogenesis when Hymenolepis was incubated with glutau-
mic acid. However α-ketoglutarate was not observed to
stimulate respiration in the worm (Read, 1956). This lack
of stimulation may have been due to permeability factors.
L-Glutamic dehydrogenase (Read, 1953) and transaminase ac-
activities have been found in the worm. It is possible that
glutamic acid could be taken up and converted to α-keto-
glutarate which could then be degraded to succinate with concomitant energy production. The succinate could serve as a substrate for terminal electron transport and glyco- genesis. The lack of appearance of \( \text{C}^{14} \) in glutamate upon \( \text{NaHCO}^{14}\text{O}_3 \) incubation and the failure to find any alpha-keto- glutarate by the methods employed would indicate that the \( \alpha \)-ketoglutaric dehydrogenase, if present, is irreversible and the rest of the citric acid cycle is absent. Thus external amino acids may serve as one of the worm's energy sources.

The results of these studies indicate that \( \text{CO}_2 \) is utilized in the synthesis of malate. This can then be converted to oxalacetate with the resulting \( \text{DENH} \) being used either for energy production via the terminal electron transport system or for reversal of the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis. Phosphoenolpyruvate resulting from oxalacetate could contribute to glucose and glycogen formation through the reversal of glycolysis. This pathway accounts for the \( \text{C}^{14} \) observed in glycogen and Read's observations that lactate was the major acid product of the worm. Fairbairn found succinate to be the major product, not lactate. At the moment it is not possible to reconcile these two observations. Possibly the difference in products is due to a difference in the carboxy- kinase activity. A drop in activity would result in the accumulation of succinate.
Read (1956) suggested that in view of the low oxygen tension of the worm's environment, an oxygen gradient from the peripheral tissues to the core of the worm might exist. In this case the malate resulting from fixation could serve as a substrate for terminal electron transport via malic dehydrogenase in the peripheral tissues where oxygen tension is greatest. In the essentially anaerobic core of the worm, where most of the glycogen and fewer of the mitochondria are located, the malate formed by fixation and other dicarboxylic acids present could be mainly involved in glycogenesis. Thus CO$_2$ fixation could serve to produce substrate for both the synthesis of high-energy phosphate and polysaccharide.

From a teleological point of view, the apparent centrality of carbon dioxide fixation is of great advantage since the worm's environment has a low oxygen tension and high carbon dioxide tension (see Read, 1950). It would appear that CO$_2$ is another of the nutrients provided by the host.

In view of the limited number of studies on carbon dioxide fixation in helminths and the apparent importance of CO$_2$ in *Hymenolepis*, *Ascaris*, and *Heterakis*, further research in this area is of great importance. Carbon dioxide may very well prove to be utilized as a nutrient by intestinal helminth parasites in general thus providing a more complete picture of the nature of the relation of the parasite to its environment, the host.
Summary

1. The in vitro incubation of *Hymenolepis diminuta* with glucose and NaHCl\(^{14}\)O\(_3\) resulted in the incorporation of C\(^{14}\) into organic acids, amino acids, and polysaccharides. This incorporation was lower under anaerobic conditions, and the incorporation into glycogen was stimulated by starvation of the worms.

2. An active phosphoenolpyruvate carboxylase was found to be present in *Hymenolepis*. The activity was very similar to that found in chicken liver.

3. A low level of malic enzyme activity was present. The enzyme had a TPN preference, but was slightly active with DPN.

4. The worm also was found to contain a quite active enzyme which appears to be different from any other fixation reaction discovered. Malate seems to be the product, and the enzyme requires coenzyme A and either manganous or magnesium ions. Other requirements and substrates have not yet been determined. The enzyme has been tentatively named the "Malate Synthesizing Enzyme."

5. The specific activity of phosphoenolpyruvate carboxy-kinase was found to be stimulated by starvation of the worm.
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